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(54) Title: STABILIZED BONE GRAFT

(57) Abstract: A demineralized bone matrix (DBM) or other matrix composition is provided that has been stabilized by lowering the pH of the composition, reducing the water content, adding water substitutes, and/or increasing the amount of deuterated water present in the composition in order to reduce the activity of endogenous degrading enzymes such as proteases. A hydrated form of a stabilized DBM composition may be stable up to a year at room temperature at acidic pH. The acidified DBM compositions may be further stabilized by the addition of a stabilizing agent such as deuterated water, water substitutes, polymers, protease inhibitors, glycerol, hydrogels, etc. The invention also provides methods of preparing, testing, and using the inventive stabilized osteodinductive matrix compositions.

STABILIZED BONE GRAFT

Related Applications

[01] The present application claims priority under 35 U.S.C. § 119(e) to U.S. provisional application, U.S.S.N. 60/539,555, filed January 27, 2004, which is incorporated herein by reference in its entirety.

Background

[02] The rapid and effective repair of bone defects caused by injury, disease, wounds, surgery, *etc.*, has long been a goal of orthopaedic surgery. Toward this end, a number of compositions and materials have been used or proposed for use in the repair of bone defects. The biological, physical, and mechanical properties of the compositions and materials are among the major factors influencing their suitability and performance in various orthopaedic applications.

[03] Autologous cancellous bone ("ACB") is considered the gold standard for bone grafts. ACB is osteoinductive, is non-immunogenic and, by definition, has all of the appropriate structural and functional characteristics appropriate for the particular recipient. Unfortunately, ACB is only available in a limited number of circumstances. Some individuals lack ACB of appropriate dimensions and quality for transplantation. Moreover, donor site morbidity can pose serious problems for patients and their physicians.

[04] Much effort has been invested in the identification or development of alternative bone graft materials. Demineralized bone matrix ("DBM") implants have been reported to be particularly useful (see, for example, U.S. Patents 4,394,370; 4,440,750; 4,485,097; 4,678,470; and 4,743,259; Mulliken *et al.*, *Calcif. Tissue Int.* 33:71, 1981; Neigel *et al.*, *Opthal. Plast. Reconstr. Surg.* 12:108, 1996; Whiteman *et al.*, *J. Hand. Surg.* 18B:487, 1993; Xiaobo *et al.*, *Clin. Orthop.* 293:360, 1993; each of which is incorporated herein by reference). Demineralized bone matrix is typically derived from cadavers. The bone is removed aseptically and/or treated to kill any infectious agents. The bone is then particulated by milling or grinding and then the mineral component is extracted (*e.g.*, by soaking the bone in an acidic solution). The remaining matrix is malleable and can be further processed and/or formed and shaped

composition; (5) adding a water substitute to the DBM composition; and/or (6) modifying covalently the osteoinductive factors in the DBM. The DBM of the stabilized DBM compositions may be provided in any form including fibers, plates, particles (*e.g.*, cubes, spheres, cones, wedges, irregular particles, *etc.*), threads, gels, *etc.* One or more of these strategies described above may be used to prepare a DBM composition with a desired shelf life, *e.g.* at least 90% of the original osteoinductive activity remaining after 1 year at room temperature.

[08] In one aspect the pH of the inventive DBM composition is lowered, the acidic environment of inventive DBM compositions slows the proteolysis of osteoinductive factors within the DBM compositions, resulting in a DBM composition with a longer shelf-life. DBM compositions with an acidic pH (*e.g.*, pH 2, 3, 4, 5, or 6) have increased stability when compared to DBM compositions at physiologic pH. In certain preferred embodiments, the acidified DBM composition retains greater than 75% of its original osteoinductivity after 6 months or 1 year of storage at room temperature. This increased shelf-life can be further extended by storing the DBM composition at a lower temperature such as 4 °C. The biological activity of acidified DBM compositions may be further stabilized by the addition of protease inhibitors (*see* U.S. patent application, U.S.S.N. 10/271,140, filed October 15, 2002; incorporated herein by reference) and/or water substitutes such as deuterated water (D_2O), DMSO, and polyols (*e.g.*, glycerol) to the composition. In addition, acidified DBM compositions may be further stabilized by the addition of stabilizing agents such as those described in USSN 10/271,140, filed October 15, 2002, incorporated herein by reference (*e.g.*, proteins, polymers, hydrogels, collagen, sugars, amino acids, lipids, *etc.*).

[09] The factors in DBM responsible for osteoinductivity are susceptible to degradation by endogenous enzymes found in the DBM, especially in hydrated DBM compositions. DBM compositions, therefore, may be stored in partially or fully dehydrated forms by lyophilizing the composition. In lyophilized form, the osteoinductivity of DBM compositions is stable even when the composition is stored at room temperature. This stabilizing effect can be further increased by storing the DBM composition at lower temperatures. Lyophilization of the DBM composition can be performed on acidified DBM compositions as well as DBM compositions with sugars,

5 years, or 10 years. In one embodiment, the pH of the inventive DBM composition is reduced by rinsing or soaking the DBM in a solution of the desired acidic pH. Optionally, the acidified DBM composition may be lyophilized to reduce the water content of the composition. Deuterated water, other water substitutes, or agents such as lipids and polymers may be added to the inventive DBM compositions during the preparation process to further stabilize and prevent the degradation of the osteoinductive agents. In certain embodiments, the composition is prepared with a carrier to make it flowable.

[13] The present invention also provides kits for preparing and using the inventive DBM compositions The kits may be used to treat bone defects using the inventive DBM compositions. For example, the DBM composition may be provided as a paste in a delivery device such as a syringe for use in a clinical setting. The DBM composition is preferably flowable for ease in using the composition. Preferably, the DBM composition is sterile and is packaged so that it can be applied under sterile or aseptic conditions (*e.g.*, in an operating room).

[14] The present invention further provides a system for characterizing DBM compositions, and for identifying and preparing DBM-containing compositions with improved properties. For example, the invention provides methods of assessing the osteoinducitivity of DBM compositions. The invention also provides methods of determining the shelf-life of DBM compositions.

Brief Description of the Drawing

[15] *Figure 1*. Comparison of the osteoinductivity of hydrated (wet) DBM and dry (lyophilized) DBM compositions at room temperature.

[16] *Figure 2*. Comparison of the osteoinductivity of hydrated (wet) and dry (lyophilized) DBM compositions with accelerated aging at 40°C.

[17] *Figure 3*.. After 5 weeks at accelerated temperatures, both non-stabilized hyaluronic acid containing samples had less than 50% of their starting osteoinductivity. The two stabilized samples had greater than 50% of their starting activity. Regression analysis were performed for all samples. The slopes (degradation rates) determined from these analysis are presented in table in Example 11. Slopes for DBM, lyophilized

[20] *Biocompatible*: The term *biocompatible* as used herein is intended to describe materials that, upon administration *in vivo*, do not induce undesirable long term effects.

[21] *Biodegradable*: As used herein, *biodegradable* materials are materials that degrade under physiological conditions to form a product that can be metabolized or excreted without damage to organs. Biodegradable materials are not necessarily hydrolytically degradable and may require enzymatic action to fully degrade. Biodegradable materials also include materials that are broken down within cells.

[22] *Demineralized bone activity* refers to the osteoinductive and/or osteoconductive activity of demineralized bone.

[23] *Demineralized bone matrix*, as used herein, refers to any material generated by removing mineral material from living bone tissue. In preferred embodiments, the DBM compositions as used herein include preparations containing less than 5% calcium and preferably less than 1% calcium by weight. Partially demineralized bone (*e.g.*, preparations with greater than 5% calcium by weight but containing less than 100% of the original starting amount of calcium) are also considered within the scope of the invention.

[24] *Diffusion barrier* refers to any material, coating, film, or substance that decreases the rate of diffusion of a substance from one side of the barrier to the other side, and more specifically, from outside to in or vice versa. The diffusion barrier in certain embodiments may be a polymer including proteins, polysaccharides, cellulose, man-made polymer, PLGA, *etc.* that prevents the diffusion of activating agents (including water, enzymes, *etc.*) and/or degradatory enzymes into the DBM composition. The diffusion barrier may also prevent the movement of osteoinductive factors out of the DBM composition. In certain embodiments, the diffusion barrier is biodegradable, leading to the degradation, activation, or release of osteoinductive factors over an extended period of time. In other embodiments, the diffusion barrier may segmentally and/or regionally degrade to control the release rates in certain regions of the composition. For a more detailed description of diffusion barriers useful in stabilizing DBM compositions, see USSN 10/271,140, filed October 15, 2002; USSN 60/392,462, filed June 27, 2002; and USSN 60/329,156, filed October 12, 2001; each of which is incorporated herein by reference.

tissue of an animal is considered osteoinductive. For example, most osteoinductive materials induce bone formation in athymic rats when assayed according to the method of Edwards *et al.* ("Osteoinduction of Human Demineralized Bone: Characterization in a Rat Model" *Clinical Orthopeadics& Rel. Res.*, 357:219-228, December 1998; incorporated herein by reference). Osteoinductivity in some instances is considered to occur through cellular recruitment and induction of the recruited cells to an osteogenic phenotype. Osteoinductivity may also be determined in tissue culture as the ability to induce an osteogenic phenotype in culture cells (primary, secondary, or explants). It is advisable to calibrate the tissue culture method with an *in vivo* ectopic bone formation assay as described by Zhang *et al.* "A quantitative assessment of osteoinductivity of human demineralized bone matrix" *J. Periodontol.* 68(11):1076-84, November 1997; incorporated herein by reference. Calibration of the *in vitro* assays against a proven *in vivo* ectopic bone formation model is critical because the ability of a compound to induce an apparent "osteogenic" phenotype in tissue culture may not always be correlated with the induction of new bone formation *in vivo*. BMP, IGF, TGF-β, parathyroid hormone (PTH), and angiogenic factors are only some of the osteoinductive factors found to recruit cells from the marrow or perivascular space to the site of injury and then cause the differentiation of these recruited cells down a line responsible for bone formation. DBM isolated from either bone or dentin have both been found to be osteoinductive materials (Ray *et al.*, "Bone implants" *J. Bone Joint Surgery* 39A:1119, 1957; Urist, "Bone: formation by autoinduction" *Science* 150:893, 1965; each of which is incorporated herein by reference).

[27] *Osteoinductivity score* refers to a score ranging from 0 to 4 as determined according to the method of Edwards *et al.* (1998) or an equivalent calibrated test. In the method of Edwards *et al.*, a score of "0" represents no new bone formation; "1" represents 1%-25% of implant involved in new bone formation; "2" represents 26-50% of implant involved in new bone formation; "3" represents 51%-75% of implant involved in new bone formation; and "4" represents >75% of implant involved in new bone formation. In most instances, the score is assessed 28 days after implantation. However, for the improved inventive formulations, particularly those with osteoinductivity comparable to the BMPs, the osteoinductive score may be obtained at earlier time points such as 7, 14, or 21 days following implantation. In these instances

or fibers may be sieved or sorted in order to collect particles of a particular size. These particles or fibers may be mixed with a solution, slurry, deformable solid, or liquid to form a paste to be used in administering or applying the graft of DBM, inventive DBM composition, or bone sample. Preferred methods of particle or fiber preparation are disclosed in issued U.S. Patents 5,607,269; 5,236,456; 5,284,655; 5,314,476; and 5,507,813; each of which is incorporated herein by reference.

[29] *Polyol* refers to any polymer having at least one hydroxyl group per repeating unit. In certain instances, a polyol has two, three, four, or more hydroxyl groups per repeating unit. Polyols may be natural or unnatural polymers. Natural polyols include polysaccharides. Unnatural polyols include polyvinyl alcohol and polyethylene glycol. Examples of polyols include polyvinyl alcohols, polyethylene glycols, erythritol, hydrogenated starch hydrolysates, isomalt, lactitol, maltitol, mannitol, sorbitol, and xylitol. In certain embodiments, polyols are used as water substitutes in DBM compositions.

[30] *Polysaccharide*, as used herein, refers to any polymer or oligomer of carbohydrate residues. The polymer may consist of anywhere from two to hundreds to thousands of sugar units. Polysaccharides may be purified from natural sources such as plants or may be synthesized *de novo* in the laboratory. Polysaccharides isolated from natural sources may be modified chemically to change their chemical or physical properties (*e.g.*, phosphorylated, cross-linked). Polysaccharides may also be either straight or branch-chained. They may contain both natural and/or unnatural carbohydrate residues. The linkage between the residues may be the typical ether linkage found in nature or may be a linkage only available to synthetic chemists. Examples of polysaccharides include cellulose, maltin, maltose, starch, modified starch, dextran, and fructose. Glycosaminoglycans are also considered polysaccharides. Sugar alcohol, as used herein, refers to any polyol such as sorbitol, mannitol, xylitol, galactitol, erythritol, inositol, ribitol, dulcitol, adonitol, arabitol, dithioerythritol, dithiothreitol, glycerol, isomalt, and hydrogenated starch hydrolysates.

[31] *Protease inhibitors*, as used herein, are chemical compounds capable of inhibiting the enzymatic activity of protein cleaving enzymes (*i.e.*, proteases). The proteases inhibited by these compounds include serine proteases, acid proteases, metalloproteases (examples of some matrix metalloprotease inhibitors are shown in

reference sample. In most cases, the reference sample will not contain the stabilizing agent, but in all other respects will be the same as the composition with stabilizing agent. The stabilizing agent also generally has little or no osteoinductivity of its own and works either by increasing the half-life of one or more of the active entities within the inventive composition as compared with an otherwise identical composition lacking the stabilizing agent. In other embodiments, the stabilizing agent may be a chemical compound that inhibits the activity of endogenous proteases or sugar-degrading enzymes. In a preferred embodiment, the stabilizing agent retards the access of one or more enzymes known to degrade one or more active factors. Half-life may be determined by immunolgical or enzymatic assay of a specific factor, either as attached to the matrix or extracted therefrom. Alternatively, measurement of an increase in osteoinductivity half-life, or measurement of the enhanced appearance of products of the osteoinductive process (*e.g.*, bone, cartilage or osteogenic cells, products or indicators thereof such as levels of gene expression associated with bone cartilage formation) is a useful indicator of stabilizing effects for an enhanced osteoinductive matrix composition. The measurement of prolonged or enhanced osteoinductive response will generally be indicative of an increase in stability of a factor. For a more detailed description of stabilizing agents useful in stabilizing DBM compositions, see USSN 10/271,140, filed October 15, 2002; USSN 60/392,462, filed June 27, 2002; and USSN 60/329,156, filed October 12, 2001; each of which is incorporated herein by reference.

[34] *Stabilizing means* refers to any manipulation of a formulation which improves its shelf-life stability either as measured at room temperature or under accelerated conditions. Preferred stabilizing means for the inventive DBM compositions include: limiting, reducing, or eliminating the availability of water to promote degradation of biological activity during storage, addition of thermodynamic stabilizers such as polyols, and the use of protease inhibitors.

[35] *Water substitute* is any liquid or flowable chemical entity which can act as a substitute for water in a DBM composition. Typically, this means to provide a desired handling quality to the DBM composition. In certain embodiments, the water substitute may be a liquid or semi-solid. A water substitute typically has hydroxyl moieties to mimic the water molecule. Preferably, the water substitute has multiple hydroxyl

processes. Available water is reduced by increasing the concentration of D_2O to inhibit endogenous degrading enzymes; and/or adding water substitutes such as glycols, polyols, hyaluronic acid, *etc.*; and/or adding water diffusion barriers. The addition of chemical and/or enzymatic inhibitors (*e.g.*, protease inhibitors) which prevent the degrading activity of hydrolysis and endogenous enzymes is also considered part of the instant invention. Water may be removed from the inventive stabilized DBM compositions to further stabilize the composition. Other methods of prolonging or stabilizing osteoinductivity may be used in conjunction with the inventive method. Preferably, the degradation of active factors within the DBM composition is inhibited to yield a desired osteoinductivity score after storage. These strategies for stabilizing DBM compositions are used to extend the shelf-life of DBM compositions so that more than 50%, generally more than 75%, and often more than 90% of the osteoinductivity of the original sample remains after one year at room temperature.

Demineralized Bone Matrix

[39] DBM preparations have been used for many years in orthopaedic medicine to promote the formation of bone. For example, DBM has found use in the repair of fractures, in the fusion of vertebrae, in joint replacement surgery, in dental surgery, and in treating bone destruction due to underlying disease such as rheumatoid arthritis. DBM is thought to promote bone formation *in vivo* by osteoconductive and osteoinductive processes. Osteoconduction occurs if the implanted material serves as a scaffold for the support of new bone growth. Osteoconduction is particularly significant when bone growth is desired across a large or "critical size" defect, across which bone healing would proceed only slowly or not at all. It is generally believed that the osteoconductive properties of DBM preparations are provided by the actual shape and coherence of the implant. Thus DBM compositions comprising entangled fibers tend to have superior osteoconductive properties as compared to less fibrous, more granular preparations. Agents, carriers, or excipients, which tend to preserve the shape and/or coherence of the DBM substituent, can lead to better bone forming properties.

[40] The osteoinductive effect of implanted DBM compositions is thought to result from the presence of active growth factors present on the isolated collagen-based

[43] Once a bone sample is obtained, it is milled, ground, pulverized, or otherwise reduced to particulate form. In preferred embodiments, the particles will be greater than 75 microns in their minimum dimension, more preferably greater than 100 microns, and more preferably greater than 150 microns. In certain embodiments, the particles are at least 200 microns across the greatest dimension. The particles may be any shape including ovals, spherical, cuboidal, cones, pyramids, wedges, coils, coiled coils, *etc.* In certain embodiments, the particles are wedges, pyramids, or cones being 200 microns across their largest dimension. In other embodiments, the DBM composition may include a mixture of several different sizes and/or shapes of particles.

[44] Following particulation, the DBM is treated to remove mineral from the bone. While hydrochloric acid is the industry-recognized demineralization agent of choice, the literature contains numerous reports of methods for preparing DBM (see, for example, Russell *et al. Orthopaedics* 22(5):524-531, May 1999; incorporated herein by reference). For the purposes of the present invention, any material that provides a scaffold containing active osteoinductive factors is considered DBM. The DBM may be prepared by any methods known in the art or by other methods that can be developed by those of ordinary skill in the art without undue experimentation. In some instances, large fragments or even whole bone may be demineralized, and then particulated following demineralization. DBM prepared in this way is within the scope of the invention.

[45] In preparing the improved DBM compositions, the DBM component may be ground or otherwise processed into particles of an appropriate size before or after demineralization. In certain embodiments, the particle size is greater than 75 microns, more preferably ranging from about 100 to about 3000 microns, and most preferably from about 200 to about 2000 microns. After grinding the DBM component to the desired size, the mixture may be sieved to select those particles of a desired size. In certain embodiments, the DBM particles may be sieved though a 50 micron sieve, more preferably a 75 micron sieve, and most preferably a 100 micron sieve.

[46] One particularly useful way to limit the access of water to the DBM is to embed the DBM in a monolithic bioabsorbable matrix, and then fragment the particle-containing monolithic matrix into particle sizes greater than 70 microns, preferably greater than 100 microns, and most preferably greater than 150 microns in their

The water in the DBM may be removed *in vacuo*, for example, the DBM may be frozen and placed in a lyophilizer under reduced pressure to remove endogenous water until the desired level of dehydration is achieved. In other embodiments, the DBM is extracted with organic solvents such as diethyl ether, tetrahydrofuran (THF), ethyl acetate, butanol, *etc.*, to remove water from the DBM. The extraction with organic solvent may be repeated to remove the desired amount of water from the matrix. The matrix may also be dehdrated by placing the matrix in a closed environment with a dessicant such as silica gel, DRI-RITE, P_2O_5, calcium carbonate, *etc.* In lyophilized or dehydrated form, the osteoinductive or osteoconductive activity of the inventive DBM composition is substantially more stable even when stored at room temperature or below over months to years. In certain preferred embodiments, the dehydrated or lyophilized DBM composition retains at least 99%, 95%, 90%, or 80% of its original activity after being stored at 37 °C for up to 1 week, up to 3 weeks, up to 5 weeks, up to 1 year, up to 2 years, or up to 3 years. In other embodiments, the lyophilized or dehydrated DBM composition retains at least 75%, 80%, 90%, 95%, 98%, or 99% of the biological activity of the original composition after 6 months, 1 year, or 2 years at room temperature or at 4 °C. Preferably, the lyophilized or dehydrated DBM composition retains at least 90% of its original biological activity after 1 year at 4 °C.

[49] *Acidification.* Endogenous proteolytic enzymes have been implicated as the source of the instability of DBM at physiologic pH. (Urist *et al. J. Histochem. & Cytochem.* 22:88-103, 1974, incorporated herein by reference). The proteolytic activity of these endogenous protease can be reduced by lowering the pH of the DBM composition. At a pH between 2-6, preferably between 3-5, and more preferably between 3-4, the inventive DBM composition has an increased stability whether in a lyophilized or dehydrated form. In certain preferred embodiments, the acidified DBM composition retains at least 99%, 95%, 90%, or 80% of its original biological activity after being stored at 37 °C for up to 1 week, up to 3 weeks, up to 5 weeks, up to 1 year, up to 2 years, or up to 3 years. In other embodiments, the acidified and/or dehydrated DBM composition retains at least 75%, 80%, 85%, 90%, 95%, 98%, or 99% of the osteoinductive or osteoconductive activity of the original composition after 6 months, 1 year, or 2 years at room temperature or at 4 °C.

temperature will increase the half-life of the biological activity of the inventive DBM composition.

[53] *Deuterated water.* Deuterated water has been shown to act as a stabilizing agent in biological compositions. For example, D_2O has been shown to increase the stability of organic molecules, macromolecules, viruses, and vaccines (Katz, "The biology of heavy water" *Scientific American* July 1960, 106-115; Katz "Chemical and biological studies with deuterium" 39th Priestly Lecture, Pennsylvania State University, 1-110; Jain *et al. Biochem. Biophys. Acta* 860:448, 1986; each of which is incorporated herein by reference). Crainic and Simpson have described increased thermal and microbial stability and slower disaggregation of certain macromolecules (published PCT application WO 94/21298; incorporated herein by reference). The presence of 95% D_2O has been found to be equivalent to a 4-5 °C reduction in storage temperature compared to H_2O, for vaccines. In addition, 7-25% D_2O helps prevent protein denaturation (*see* Wenzel, DE2253086; Hamaya and Horikoshi, JP01179689; Teva Pharm. Industries, Ltd. EP 332826; each of which is incorporated herein by reference). The present invention demonstrates that the addition of deuterated water to DBM or the soaking or rinsing of DBM in deuterated water results in the stabilization of the osteoinductivity of DBM compositions. In certain embodiments, the DBM is dehydrated to remove H_2O and then re-hydrated with D_2O. Preferably, the percentage of D_2O is greater than 50%, more preferably greater than 75%, and even more preferably greater than 90%. In certain embodiments, the percentage of D_2O is greater than 95%. However, in some embodiments, the percentage of D_2O may be 5-10%, 10-20%, 20-30%, or 40-50%. In other embodiments, the pD of a D_2O solution or buffer used to prepare a DBM compositions is below 7.0, preferably between 2 and 5, and more preferably between 3 and 4. By lowering the pD of the D_2O solution or buffer, the osteoinductivity is further stabilized by reducing the activity of endogenous proteases as discussed above. The use of D_2O as a water substitute may also be combined with other strategies for stabilizing DBM compositions such as adding a stabilizing agent such as a sugar, adding a protease inhibitor(s), and adding other water substitutes as described below.

[54] *Other water substitutes.* Water substitutes may also inhibit chemical reactions in which water participates, or water is the required medium for the reaction. Water

higher fatty acids such as lauric acid, myristic acid, palmitic acid, stearic acid, behenic acid, and arachidic acid.

[57] The addition of water substitutes to a composition also has the benefit of making the composition flowable and moldable.

[58] *Addition of stabilizing agents.* The incorporation of stabilizing agents into the inventive formulations is generally accomplished by suspending the molecule or molecules of interest in an appropriately compatible buffer as will be known to those skilled in the art. This buffer is then mixed with matrix in a relatively low liquid-to-solid volume ratio to form a slurry. Preferably, the pH of the buffer is approximately pH 7.4. In embodiments where the composition is acidified, the pH of the buffer is less than physiological pH is approximately the pH desired in the final DBM composition. In certain embodiments, the buffer with the stabilizing agent(s) is mixed with lyophilized matrix. The slurry may then be lyophilized and used to prepare the desired DBM formulations.

[59] One feature of the instant invention is that the incorporation of diffusion barriers, enzyme inhibitors (*e.g.*, protease inhibitors), competitive substrates, masking agents, or other stabilizing agents often has the additional feature of further improving the DBM formulation's shelf-life by preventing access of endogenously present degradative enzymes to the active factors present in the matrix. This is particularly true for DBM formulations which are prepared containing water (*e.g.*, DBM preparations with hydrogel carriers such as hyaluronic acid or collagen, or hydrated starch carriers).

[60] Diffusion barriers retard the diffusion of degradative enzymes and/or water to the active moieties within the inventive formulations. Enzymes retarded in their diffusion to the included DBM may be capable of releasing the active factor from the matrix, and/or degrading or inactivating the active factor. They also may act by retarding diffusion of the active factors from the implant site. In these ways, the barriers provide for longer residence time of the active factors at the implant site. This is particularly useful for forming bone in higher species such as humans, where bone formation appears to require the presence of active factors for longer times.

[61] Generally, materials most suitable to serve as diffusion barriers will be easily mixed with DBM or synthetic matrix of choice to form a gel, paste, or putty-like consistency, although in some embodiments, the barrier/matrix formulation will be

important consideration in designing the inventive formulations. Those skilled in the art are aware of the variety of amylase susceptibilities of starches prepared from various plant sources and may apply this knowledge to produce formulations having a desired stability time. Preferred starches will degrade as much as 10% per day, preferably 50% per day, and most preferably greater than 90% per day. Those starches less susceptible to degradation by pectinase and/or amylase (amylase-resistant starch; Starch Australasia, Sydney, Australia) may be used to maximally extend the osteoinductive half-life *in vivo* to an even greater extent than improved DBM or synthetic growth factor/matrix formulations prepared from more enzyme susceptible starches. Some modified starches are less susceptible to degradation by amylase; therefore, improved DBM with modified starch would presumably have a longer half-life *in vivo* as compared to those improved DBM with unmodified starch. One preferred method to affect amylase susceptibility of starch is through the use of starch lipid combinations. Guidance for the combination of lipid and starch to affect amylase susceptibility is given by Crowe *et al.* "Inhibition of Enzymic Digestion of Amylose by Free Fatty Acids In Vitro Contributes to Resistant Starch Formation" *J. Nutr.* 130(8):2006-2008, August 2000; incorporated herein by reference. Similar considerations apply to lipids and their degradative enzymes the lipases. A large variety of mono-, di-, and triglycerides with varying degrees of susceptibility to lipase degradation are available from commercial sources. Some embodiments include one or more polymeric materials, preferably biodegradable, such as tyrosine polycarbonates, polyfumarates, tyrosine polyarylates, and poly-orthoesters such as polylactide, polygalactide, and co-polymers thereof. These polymers are biodegradable, and their properties can be modified by altering the chain length or degree of cross-linking of the polymer and/or the chemical structure of the monomers. Additionally, co-polymers can be prepared using combinations of resorbable polymers.

[64] Enzyme inhibitors useful in the practice of the present invention may include, for example, acid protease inhibitors, serine protease inhibitors, metalloprotease inhibitors (*see* Whittaker *et al.* "Matrix Metalloproteinases and their Inhibitors-Current Status and Future Challenges" *Celltransmissions* 17(1):3-14; incorporated herein by reference), cysteine protease inhibitors, glyconase inhibitors, and glycosidase inhibitors. Specific protease inhibitors useful in the practice of the present invention

[68] *Exemplary stabilized DBM compositions*. Based on the above strategies for stabilizing DBM compositions and those discussed in other applications which are incorporated herein by reference, the following DBM compositions are particularly useful:

[69] Certain DBM compositions do not include glycerol. In certain embodiments, the DBM composition comprises DBM and a non-glycerol stabilizing means. The stabilizing means may include a water substitute such as deuterated water, polyols, lipids, oils, waxes, polysaccharides, *etc.*; protease inhibitors; acids; diffusion barriers; competitive substrates; masking agents; and covalent modifications. In certain particular embodiments, the stabilizing means is a water substitute, protease inhibitor, acid, or a diffusion barrier. Particularly useful water substitutes are D_2O and non-glycerol polyols. In certain embodiments, the DBM composition is acidified, and a water substitute is added. In other embodiments, the DBM compositions is acidified and a protease inhibitor is added. In yet other embodiments, the DBM composition includes a water substitute and a protease inhibitor. Water from the DBM composition may optionally be removed by lyophilization or other means as described herein. The DBM composition should preferably retain at least 95% of its original osteoinductive activity after 1 year at room temperature.

[70] The inventive DBM composition may further comprise a non-glycerol carrier. Non-glycerol carriers useful in the include DBM compositions include hyaluronic acid, collagen, lipid, polymers, and water. A carrier may be added to the DBM composition to make the composition more flowable, easier to mold, and/or easier to work with. Preferably, the addition of a carrier does not substantially affect the stability or half-life of the DBM composition.

[71] In certain embodiments, the DBM composition include glycerol as a water substitute. In certain embodiments, the DBM composition includes DBM, glycerol, and stabilizing means. The stabilizing means may be a water substitute, acidification, diffusion barrier, or protease inhibitor. Examples of particularly useful stabilizing means include hyaluronic acid, starch, and lipid.

[72] In certain embodiments, the DBM is mixed with an exogenous destabilizing entity. For example, the DBM may be mixed with a tissue containing proteases or other degradatory enzymes. In order to protect the active factors in the DBM from

incubation), the packages are opened and the osteoinductive activity of the contents is determined . The accelerated stability of any sample can be determined from the slope of the best fit line obtained by plotting the osteoinductive activity of each DBM formulation, normalized to percent initial osteoinductive activity, against time. The room temperature stability of the materials could be extrapolated using Von't Hoff's rule of enzyme kinetics as described by Reick *et al.*, *Medical Device and Diagnostic Industry* 10(3):34-39, 1998; incorporated herein by reference.

[76] In our studies as shown below in the Examples, acidifying the DBM composition or adding glycerol to a hydrated a DBM formulation, significantly increases the osteoinductive stability of DBM.

Measurement of Osteoinductive Activity

[77] Osteoinductive activity is determined by implanting the DBM formulation of interest in a nonskeletal site in an athymic rat and evaluating the amount of new bone, cartilage, and bone marrow that is induced at the site of the implant. The procedure for determination of osteoinductive activity has previously been described in detail (Edwards JT, Diegmann MH, Scarborough NL. "Osteoinduction of human demineralized bone: characterization in a rat model" *Clin. Orthop.* 1998 Dec; (357):219-28; incorporated herein by reference).

Osteoinducer

[78] To the DBM composition may be added other osteoinducing agents. These agents may be added in an activated or non-activated form. These agents may be added at anytime during the preparation of the inventive material. For example, the osteoinducing agent may be added after the demineralization step and prior to the addition of the stabilizing agents so that the added osteoinducing agent is protected from exogenous degrading enzymes once implanted. In some embodiments the DBM is lyophilized in a solution containing the osteoinducing agent. In certain other preferred embodiments, the osteoinducing agents are adhered onto the hydrated demineralized bone matrix and are not freely soluble. In other instances, the osteoinducing agent is added to the improved DBM after addition of the stabilizing

DBM composition with a desired half-life. Alternatively or additionally, the particle size may be important in determining the half-life of the inventive DBM composition. In certain preferred embodiments, an inventive formulation may include a mixture of particles, each with a different half-life. Such a mixture could provide the steady or possible unmasking of osteoinductive factors over an extended period of time ranging from days to weeks to months depending on the needs of the injury. Compositions such as this can be formulated to stimulate bone growth in a human patient comparable to the bone growth induced by treatment with 10 μg of rhBMP on a collagen sponge, and preferably comparable to 100 μg, and most preferably 1-10 mg rhBMP.

[82] Physical properties such as deformability and viscosity of the DBM may also be chosen depending on the particular clinical application. The particles of the improved DBM may be mixed with other materials and factors to improve other characteristics of the implant. For example, the improved DBM material may be mixed with other agents to improve wound healing. These agents may include drugs, proteins, peptides, polynucleotides, solvents, chemical compounds, biological molecules, *etc.*

[83] The particles of DBM (or inventive DBM material) may also be formed into various shapes and configurations. The particles can be formed into rods, strings, sheets, weaves, solids, cones, discs, fibers, wedges, coils, coiled coils, *etc.* In certain embodiments, the shape and size of the particles in the DBM composition affect the time course of osteoinductivity. For example, in a cone or wedge shape, the tapered end will result in osteoinductivity shortly after implantation of the DBM composition, whereas the thicker end will lead to osteoinductivity later in the healing process (*e.g.*, hours to days to weeks later). In certain embodiments, the particle have a length of greater than 2 mm, greater than 1.5 mm, greater than 1 mm, preferably greater than 500 microns, and most preferably greater than 200 microns across its widest dimension. Also, larger particle size will have induced bone formation over a longer time course than smaller particles. Particles of different characteristics (*e.g.*, composition, size, shape) may be used in the formation of these different shapes and configurations. For example, in a sheet of DBM a layer of long half-life particles may be alternated between layers of shorter half-life particles (see U.S. Patent 5,899,939, incorporated herein by reference). In a weave, strands composed of short half-life particles may be woven together with strands of longer half-lives.

produce bone in human patients with similar timing and at a level similar to 10 μg to 100 μg, preferably 200 μg to 1 mg of rhBMP on a collagen sponge. For example, specific bones that can be repaired using the inventive material include the ethmoid, frontal, nasal, occipital, parietal, temporal, mandible, maxilla, zygomatic, incus, stapes, malleus, cervical vertebrae, thoracic vertebrae, lumbar vertebrae, sacrum, sternum, ribs, clavicle, scapula, humerus, ulna, radius, carpal bones, metacarpal bones, phalanges, ileum, ischium, pubis, pelvis, femur, patella, tibia, fibula, calcaneus, talus, and metatarsal bones. The type of injury amenable to treatment with the improved DBM include bone defects resulting from injury, brought about during the course of surgery, infection, malignancy, or developmental malformation. The inventive material may be useful in orthopaedic, neurosurgical, cosmetic, and oral and maxillofacial surgical procedures such as the repair of simple and compound fractures and non-unions, external and internal fixations, joint reconstructions such as arthrodesis, general arthroplasty, cup arthroplasty of the hip, femoral and humeral head replacement, femoral head surface replacement and total joint replacement, repairs of the vertebral column including spinal fusion and internal fixation, tumor surgery (*e.g.*, deficit filling), discectomy, laminectomy, excision of spinal cord tumors, anterior cervical and thoracic operations, repair of spinal injuries, scoliosis, lordosis and kyphosis treatments, intermaxillary fixation of fractures, mentoplasty, temporomandibular joint replacement, alveolar ridge augmentation and reconstruction, inlay bone grafts, implant placement and revision, sinus lifts, *etc.*

[88] These and other aspects of the present invention will be further appreciated upon consideration of the following Examples, which are intended to illustrate certain particular embodiments of the invention but are not intended to limit its scope, as defined by the claims.

Examples

Example 1: Preparing Demineralized Bone Matrix (DBM)

[89] DBM may be prepared using any method or technique known in the art (see Russell *et al. Orthopedics* 22(5):524-531, May 1999; incorporated herein by reference).

of ether per gram of starting bone particles. After the last change of ether is removed, the demineralized bone powder is left overnight in the hood until all the residual ether has vaporized. The particles should be odorless, snow-white, and discrete. To sterilize the demineralized bone material, it may be treated with cold ethylene oxide or irradiated.

[93] To test the bioactivity of the prepared DBM, 25 mg of the material is implanted into each of two thoracic subcutaneous pockets in shaved, anesthetized 28-day old male Charles River CD rats. The implanted specimens may then be harvested and inspected several days after implantation. The composition of the induced tissue can be quantified by histomorphometric analysis and biochemical techniques.

Example 2: Another Method of Preparing DBM.

[94] DBM may be prepared using any method or techniques known in the art (See Russell *et al. Orthopedics* 22(5):524-531, May 1999; incorporated herein by reference).

[95] Demineralized bone matrix was prepared from long bones. The diaphyseal region was cleaned of any adhering soft tissue and then ground in a mill. Ground material was sieved to yield a powder with particles approximately 100 μm to 500 μm in diameter. The particulate bone was demineralized to less than about 1% (by weight) residual calcium using a solution of Triton X-100 (Sigma Chemical Company, St Louis, MO) and 0.6N HCl at room temperature followed by a solution of fresh 0.6N HCl. The powder material was rinsed with deionized water until the pH was greater than 3.0. It then was soaked in 70% ethanol and freeze-dried to less than 5% residual moisture.

Example 3: *In vitro* assessment of pH-stabilized DBM

[96] Samples of pH stabilized DBM with or without stabilizing agents (or various concentrations and/or formulations of stabilizing agents) are prepared and incubated with serum or individual enzymes (*e.g.*, papain) in PBS buffer (pH 7.4) and incubated at 37 °C for 0.5, 1, 2, 4, 8, and 24 hours Samples are then extracted to determine the concentrations of growth factors and other matrix proteins as outlined in Ueland *et. al.* ("Increased cortical bone content of insulin-like growth factors in acromegalic patients" *J. Clin. Endocrinol. Metab.* 1999 Jan;84(1):123-7; incorporated herein by reference).

muscle of each rat. Animals are allowed normal activities following surgical procedures.

[102] *Implant Materials*: DBM and test materials are kept at room temperature. Samples are tested for implantation times of 7, 14, and 28 days. Samples of DBM powder are rehydrated with 100 µl of sterile ALLOPREP™ (Ostetotech, Eatontown, NJ). Each of the samples is packed into a 1 ml blunt cut syringe. Implantation is randomized so that a single animal does not receive two of the same implants.

[103] *Anesthesia*: The rats are anesthetized with a mixture of ketamine (200 mg), xylazine (400 mg), and physiological saline (10 ml). The dosage is 3.5 ml/kg body weight administered intraperitoneally.

[104] *Procedure:* Aseptic surgical procedures are carried out in a laminar airflow hood. A 1-cm skin incision is made on each upper hind limb using a lateral approach, and the skin is separated from the muscle by blunt dissection. A superficial incision aligned with the muscle plane is made to allow for insertion of the tips of the scissors. Blunt dissection is performed from this line deep into the muscle to create a pocket to hold the implanted material. A single suture is inserted to close the muscle pocket, and the skin is closed with metal clips.

[105] Implantation of specimens in the left pectoralis muscles involves making a 1-cm skin incision over the chest, blunt dissection of the muscle to create a pocket, and positioning of the rat DBM powder using a blunt syringe. A single suture is inserted to close the muscle pocket, and the skin is closed with metal clips.

[106] Rats are euthanized with CO_2 following the designated implantation time. Implant materials are located by palpitation, retrieved by blunt dissection, and cleaned of the surrounding tissue by careful trimming. An observer blinded to implant type performed a macroscopic evaluation of the implant material. Color, vascularity, hardness, and integrity are scored according to the scheme outlined in the Table below. (The highest score for the most robust response would be a 4 while a specimen showing little or no osteoinductive potential would score a 0.) Experience with this model has shown a high correlation between visual observations and histological observations of implant performance only at the extremes of both ends of the scale.

Macroscopic Observation Scoring Guidelines

Color:	White (W)	Grey (G)	Red (R)

paravertebral muscle following a protocol specified procedure. Animals assigned to the High Dose treatment group (n=20) receive 3.5 ml of the test article in the right paravertebral muscle and 7.0 ml of the test article in the subcutaneous tissue of each side of the dorsal thoracic area. The animals assigned to the Control treatment group (n=15) are implanted with 3.5 ml of control article (rehydrated DBM powder) in the right paravertebral muscle. At 7, 14, and 28 days post-implantation, four animals from the Low and High Dose treatment groups and three animals from the Control groups are humanely sacrificed. At 60 days post-implantation, the remaining animals are sacrificed (eight from the Low and High Dose test groups and six from the Control treatment group). The implant sites are collected from each rabbit and fixed in 10% neutral buffered formalin (NBF). The test and control implant sites from the 60 days post-implantation study interval are placed in decalcification solutions for 3 days after adequate formalin fixation. All tissue samples are processed using standard histological techniques, sectioned at 5 μm, and stained with hematoxylin and eosin.

Example 6: Terminal Sterilization

[111] This example describes a terminal sterilization method which minimizes osteoinductivity loss in the inventive preparations.

[112] The inventive DBM preparations are produced in a clean room environment from human tissue. The finished implants are placed in individual tray packages.

[113] Each tray is placed in an Audionvac sealing apparatus (Audion Electro B.V., Weesp-Holland) which is supplied with a cylinder consisting of 50/50 hydrogen/argon gas. Before the tray packages are sealed, they are evacuated and backfilled with the gas mixture twice. Following sealing, the gas mixture remains in each tray package.

[114] The packaged implants are then sealed packages and then treated with 15 KGy gamma radiation from a cobalt 60 source to reduce the bioburden of the implants to the desired level.

Example 7: Storage of acidic DBM at Room Temperature

[115] To determine the stability of acidic DBM in real time, a DBM composition comprising pooled human demineralized bone powder (100-500 micron particle size) at pH ~5.0 was prepared and subjected to storage at room temperature. Sixteen samples

equivalent of 3 years (based on accelerated aging). In contrast, DBM samples which were stored in the hydrated form lost about 50% of their osteoinductivity in the same time frame (based on accelerated aging) (Figure 8). These data indicate that while both storage method, *i.e.*, hydrated or lyophilized retain the osteoinductivity of the acidic DBM, a lyophilized acidic DBM is more stable and retains at least 75% of its osteoinductivity up to three years.

Real Time Equivalent	0 weeks	6 months	12 months	24 months	36 months
Hydrated DBM	4	3.25	2	2	1.5
Lyophilized DBM	4	3.25	3.2	2.75	3

Example 9: Storage of Acidic DBM plus Starch Carrier at 40°C

[117] To assess the stability of an acidic DBM containing a starch carrier, a DBM comprising pooled human demineralized bone powder (100-500 micron particle size) and a carrier (maltodextrin, modified starch, and water) at pH ~4.3 was made and subjected to accelerated aging at 40°C. Samples were packaged and sealed in foil packages. Accelerated aging testing was done at 40°C ± 2°C, 75% ± 5% relative humidity. Eight samples were prepared for each time point and stored in hydrated form. Samples were packed in foil pouches, sealed and stored at 40°C ± 2°C, 75% ± 5% relative humidity, for 0, 5.3, 10.6, 21.2, and 31.8 weeks until implanted. Storage at 40°C for the times indicated is equivalent to room temperature, real time storage up to 0, 6, 12, 24,and 36 months, respectively (based on Q_{10}=2). Each accelerated-aging sample had a real time sample analyzed in parallel after 0, 6, 12, 24, and 36 months of aging at room temperature. Samples were implanted inter-muscularly in the upper thigh of female nude rats as described in Edwards *et al. Clinical Orthopaedics* 357:219-228, 1998, incorporated herein by reference. The DBM was subsequently explanted 28 days after surgery for histological evaluation and scoring of osteoinductivity as described in the above reference. Results of histological studies showed that there was only a slight difference between samples tested under real time and the real time equivalent of accelerated aging conditions. These data indicate that an acidic DBM containing a starch carrier retains more than 75% and about 80% of its osteoinductivity after three years. This also demonstrates that there is a correlation between real time aging of DBM and accelerated aging.

herein by reference. The DBM is subsequently explanted 28 days after surgery for histological evaluation and scoring of osteoinductivity as described in the above reference. Results of histological studies are expected to show that the addition of a hyaluronic acid carrier to a pH neutral DBM maintains osteoinductive stability for at least 1 year or more while the osteoinductivity of pH neutral DBM is less stable.

[120] Results: After 5 weeks at accelerated temperatures, both non-stabilized hyaluronic acid containing samples had less than 50% of their starting osteoinductivity (see Figure 3). The two stabilized samples had greater than 50% of their starting activity. Regression analysis were performed for all samples. The slopes (degradation rates) determined from these analysis are presented in the table below . Slopes for DBM, lyophilized or in the presence of water determined in previous studies, are included in the table for comparison purposes.

Degradation Rates of Various Demineralized Bone Preparations at 40 °C

Preparation	Degradation rate (%OI/week)
Lyophilized DBM	0.36-0.52
Lyophillized DBM plus glycerol	0.58
DBM hydrated with water (pH<5)	1.4
HA + glycerol	6.7
HA + acid	7.2
HA-1	15.3
HA-2	16.9

Example 12: Stabilization of a Neutral DBM with a Collagen Carrier

[121] To evaluate the stability of a neutral DBM composition which has been pH stabilized with the addition of a collagen carrier, a neutral pH DBM comprising pooled human demineralized bone powder (100-500 micron particle size) is made with and without a collagen carrier and subjected to accelerated aging. Accelerated aging testing is done at 40°C ± 2°C, 75% ± 5% relative humidity. Samples are prepared with and without a collagen carrier for each time point and stored in hydrated form. Test samples are packed in foil pouches, sealed, and stored at 40°C ± 2°C, 75% ± 5% relative humidity, for 0, 5.3, 10.6, 21.2, and 31.8 weeks until implanted. Storage at 40

20 g DBM	10 g Hyaluronic acid	100 cc10% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 20% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 30% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 40% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 50% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 60% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 70% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 80% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 90% Glycerol
20 g DBM	10 g Hyaluronic acid	100 cc 99% Glycerol
20 g DBM	10 g B-980 Starch	60 cc 40% Dimethysulfoxide
20 g DBM	10 g B-980 Starch	60 cc 1.5 M Glycine
20 g DBM	10 g B-980 Starch	60 cc 2.5 M Proline
20 g DBM	10 g B-980 Starch	60 cc 1.2 M Sucrose
20 g DBM	10 g B-980 Starch	60 cc 1.0 M Trehalose
20 g DBM	10 g B-980 Starch	60 cc 200 mM K_2PO_4
20 g DBM	10 grams Hydrated Carrier (starch, *etc.*)	5 grams hydrophobic lipid (tripalmitate, cholesterol, *etc*)
20 g DBM	10 g Hyaluronic acid	60 cc 40% Dimethysulfoxide
20 g DBM	10 g Hyaluronic acid	60 cc 1.5 M Glycine
20 g DBM	10 g Hyaluronic acid	60 cc 2.5 M Proline
20 g DBM	10 g Hyaluronic acid	60 cc 1.2 M Sucrose
20 g DBM	10 g Hyaluronic acid	60 cc 1.0 M Trehalose
20 g DBM	10 g Hyaluronic acid	60 cc 200 mM K_2PO_4
20 g DBM	10 g Hyaluronic acid	5 grams hydrophobic lipid (tripalmitate, cholesterol, *etc.*)
20 g DBM	10 grams non DBM protease containing tissue (mineralized bone, *etc.*)	100 cc (1-99 %) Glycerol
20 g DBM	10 grams non DBM protease containing tissue (mineralized bone	20 g hydrophobic lipid (tripalmitate, cholesterol, *etc.*)
20 g DBM	10 grams non DBM protease containing tissue (mineralized bone	60 cc 2 mM N-ethylmaleimide
20 g DBM	10 grams non DBM protease containing tissue (mineralized bone	60 cc 0.1 mM 4-(2-Aminoethyl)benzenesulfonylfluoride HCl
20 g DBM	10 grams hydrated non DBM protease containing tissue (mineralized bone	Heat treatment at 59 °C for 2 hrs

[124] Stability testing. Samples are packaged in moisture resistant containers (aluminum foil) and placed at room temperature for a period of time exceeding 3 years. At various time periods (*e.g.*, 1 month, 3 months, 6 months, 1 year, 2 years, 3 years, 4

Claims

1. A demineralized bone matrix composition comprising:

demineralized bone matrix;

at least one non-glycercol stabilizing means;

wherein the composition retains at least 50% of its original osteoinductivity after one year at room temperature.

2. The composition of claim 1, wherein the composition does not include glycerol.

3. The composition of claim 1, wherein the demineralized bone matrix is in the form selected from the group consisting fibers, plates, particles, threads, and gels.

4. The composition of claim 1, wherein the non-glycerol stabilizing means is selected from the group consisting of deuterated water (D_2O), protease inhibitors, non-glycerol polyols, polysaccharides, and acids.

5. The composition of claim 1 further comprising water.

6. The composition of claim 1 further comprising hyaluronic acid.

7. The composition of claim 1, wherein the non-glyercol stabilizing means is a protease inhibitor or combination of protease inhibitors.

8. The composition of claim 7, wherein the protease inhibitor is selected from the group consisting of aprotinin, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), amastatin-HCl, alpha1-antichymotrypsin, antithrombin III, alpha1-antitrypsin, 4-aminophenylmethane sulfonyl-fluoride (APMSF), arphamenine A, arphamenine B, E-64, bestatin, CA-074, CA-074-Me, calpain inhibitor I, calpain inhibitor II, cathepsin inhibitor, chymostatin, diisopropylfluorophosphate (DFP), dipeptidylpeptidase IV inhibitor, diprotin A, E-64c, E-64d, E-64, ebelactone A, ebelactone B, EGTA, elastatinal, foroxymithine, hirudin, leuhistin, leupeptin, alpha2-macroglobulin,

18. The composition of claim 1, wherein the composition retains at least 75% of its original osteoinductivity after 2 years at room temperature.

19. The composition of claim 1, wherein the composition retains at least 90% of its original osteoinductivity after 2 years at room temperature.

20. The composition of claim 1 further comprising at least one exogenous osteoinductive or osteogenic agent.

21. A deminerlized bone composition comprising:

demineralized bone matrix;

a non-glycerol carrier; and

a stabilizing means,

wherein the composition retains at least 90% biological activity after one year.

22. The composition of claim 21, wherein the demineralized bone matrix is in the form selected from the group consisting fibers, plates, particles, threads, and gels.

23. The composition of claim 21, wherein the carrier is selected from the group consisting of hyaluronic acid, collagens, lipids, polymers, proteins, and water.

24. The composition of claim 21, wherein the carrier is selected from the group consisting of hyaluronic acid, collagens, lipids, polymers, and water.

25. The composition of claim 21, wherein the stabilizing means is selected from the group consisting of deuterated water (D_2O), protease inhibitors, non-glycerol polyols, sorbitol, and acids.

26. A demineralized bone matrix composition comprising:

a demineralized bone matrix;

glycerol; and

32. A kit comprising the demineralized bone matrix composition of claim 1 conveniently packaged for clinical use.

33. The kit of claim 32 wherein the demineralized bone matrix is packaged in sterile form.

34. The kit of claim 32 wherein the demineralized bone matrix is packaged in a syringe.

Real Time Wet v.s. Dry

n=8 for each point as per Michelle

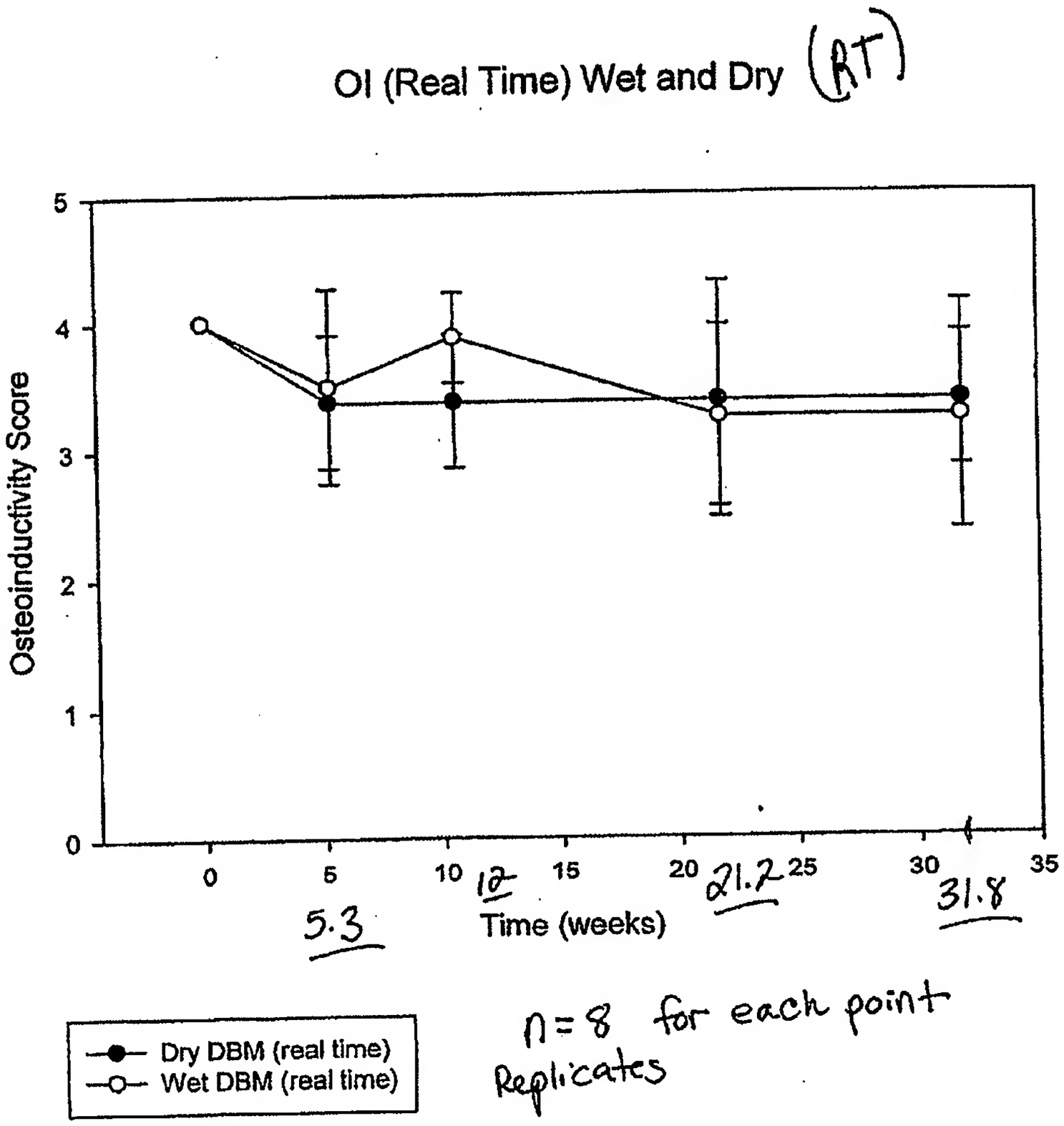
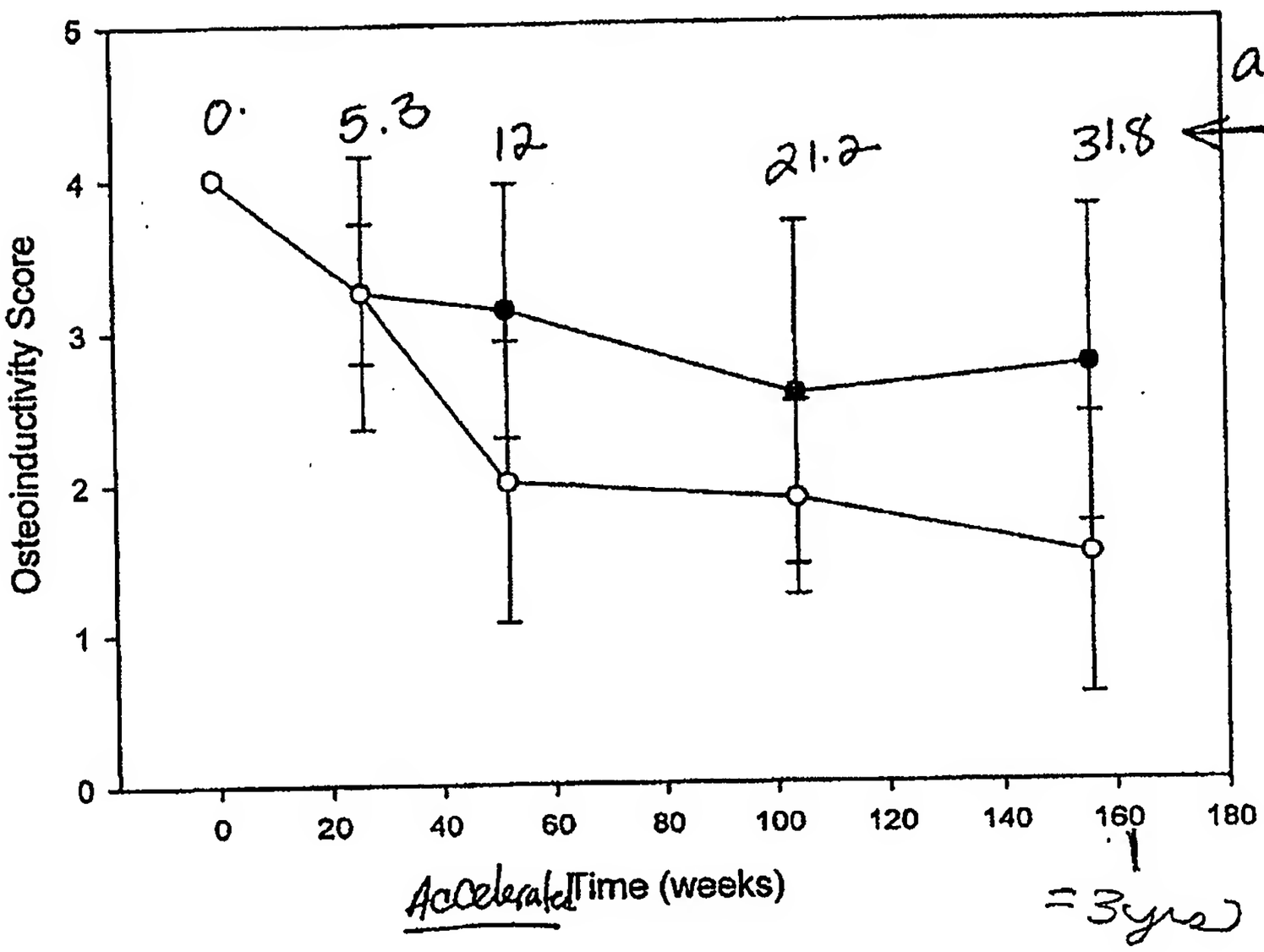


FIG. 1

Accelerated Only.
Wet & Dry DBM.

01 (Accelerated) Wet + Dry

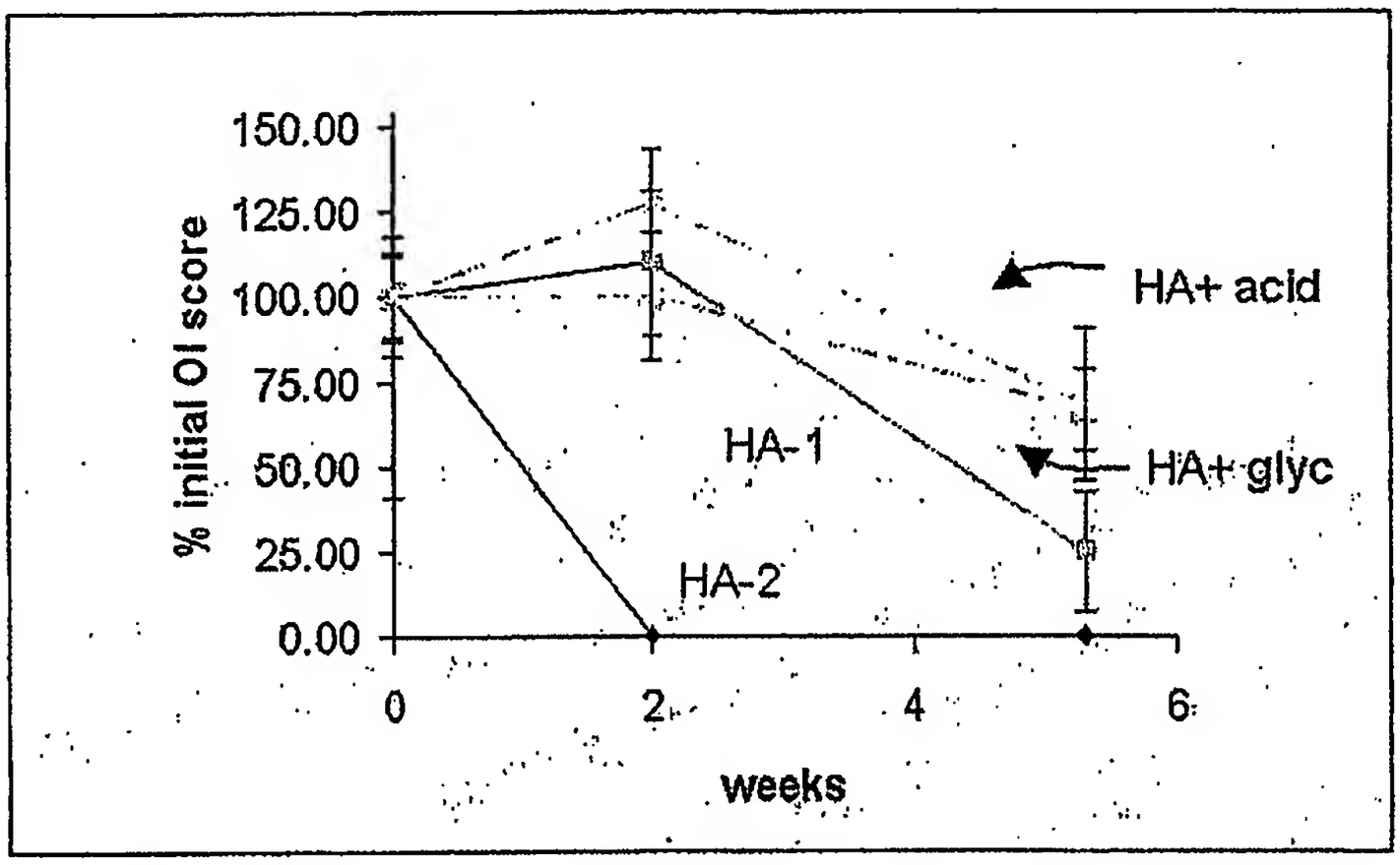


n=8 for each point
Replicates

Accelerated Arg.
@ RT = 40° equivalent
days
5.3 ⇒ 6 mos
10.2 ⇒ 12 mos
? 21.8 OR ⇒ 21.8 ⇒ 24 mos
31.8 ⇒ 36 mos

FIG. 2

Figure 3


150.00
125.00
100.00
75.00
50.00
25.00
0.00
% initial OI score
0
2
4
6
weeks
HA-1
HA-2
HA+ acid
HA+ glyc

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/03092

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61F 2/28
US CL : 424/426; 523/114,115
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/426; 523/114,115

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,468,543 A (GILBERTSON et al) 22 October 2002, see column 11, lines 26-38, 45-67; column 12, lines 5-10; column 13, lines 16-44.	1-4, 9-24, 27, 31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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